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(54) Title: WT1 MONOCLONAL ANTIBODIES AND METHODS OF USE THEREFOR		
(57) Abstract <p>The present invention provides three unique monoclonal antibodies directed against a portion of the Wilms' tumor antigen, and methods of use therefor in detecting, monitoring and diagnosing malignancies characterized by over-expression or inappropriate expression of the WT1 protein.</p>		

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WT1 MONOCLONAL ANTIBODIES AND METHODS OF USE THEREFOR

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Field of the Invention

This invention relates generally to the field of
10 detecting, monitoring and diagnosing malignancies characterized by expression of the Wilms' tumor 1 antigen.

Background of the Invention

15 The Wilms' tumor (wt1) gene encodes a protein referred to as WT1 which is expressed in the nucleus of certain cells and possesses the structural features of a DNA binding transcription factor. As illustrated in Fig. 1 below, the WT1 protein is a 429 amino acid protein [SEQ
20 ID NO:4] which contains four contiguous zinc fingers at the carboxyl-terminus, and a glutamine- and proline-rich region at the amino-terminus. The amino-terminal region of WT1 protein mediates transcriptional suppression or activation in transient transfection assays [Madden et
25 al, Science, 253:1550-1553 (1991); Maheswaran et al, Proc. Natl. Acad. Sci. USA, 90:5100-5104 (1993); S. L. Madden et al, Oncogene, 8:1713-1720 (1993)]. Splice variants of WT1 can produce the protein with a 17 amino acid insert at amino acid 249 and/or a 3 amino acid
30 insert at amino acid 390.

The wt1 gene encoding WT1 protein is located on chromosome 11p13 and has been found to be mutated or deleted in a subset of hereditary and sporadic Wilms' tumors. Recently, high levels of wt1 expression were
35 reported in a variety of tumors such as ovarian carcinomas [Bruenig et al, Cancer Invest., 11:393-399

(1993)], prostate cancer, mesotheliomas [Park et al, cited above], and leukemias [Miwa et al, Leukemia, 6:405-409 (1992), Miyagi et al, Leukemia, 7:970-977 (1992)].

Diagnostic methods for the ovarian carcinomas, mesotheliomas, and leukemias referred to above are based primarily on clinical attributes and histology of tumor specimens. These methods may at times not distinguish between closely related diseases and may lead to inappropriate treatments of patients. For example, in addition to the presence of many histological variants of malignant mesothelioma, there are other lesions that can affect the pleural surface and present a clinical and histological picture quite similar to malignant mesothelioma [R. J. Pisani et al, Mayo Clin. Proc., 63:1234-1244 (1988)]. Additional relatively specific molecular markers that clearly distinguish between clinically similar lesions for malignant mesotheliomas as well as the other cancers would thus be a valuable clinico-pathological tool which will permit a precise diagnosis. This is important since treatment protocols and prognosis for such conditions vary significantly.

Currently available diagnostic tools include rabbit polyclonal antibodies for WT1 protein known in the art. Morris et al, Oncogene, 6:2339-2348 (1991) describe two such antibodies which recognize amino acid fragments spanning amino acids 294-429 of SEQ ID NO:4 and amino acids 85-173 of SEQ ID NO:4, respectively, of the WT1 protein. Another rabbit polyclonal antibody, which recognizes WT1 amino acids 275-429 of SEQ ID NO: 4 was described by Telerman et al, Oncogene, 7:2545-2548 (1992). Still other WT1 polyclonal antibodies are commercially available, e.g. the rabbit polyclonal antibody SC-192, which is available from Santa Cruz. However, while polyclonal antibodies in general are able to detect WT1 expression, they have disadvantages in

their potential for cross-reactivity with closely related proteins which share common domains with the WT1 protein. These polyclonal antibodies by their nature are likely to provide inconsistent results in antigen specificity and binding affinity studies and are not particularly desirable for diagnostic uses.

Additionally, a commercially available mouse monoclonal antibody, DG-10 (Applied BioTechnology) was raised to the zinc finger region of WT1 and is known to cross-react with the Egr1 proteins. Expression of Egr1 proteins is not limited to cells or tissues that express WT1 and is independently regulated from WT1 expression. Therefore, any antibodies raised to the zinc finger domain in the carboxyl terminus of WT1 may not be useful for selective detection of the WT1.

Another anti-WT1 mouse monoclonal antibody has been described by Mundlos et al, Development, 119:1329-1341 (1993). The Mundlos et al antibody is specific for a 17 amino acid sequence insert (See Fig. 1 below), i.e., a splice variant, that is present in only a subpopulation of the alternatively spliced WT1 mRNA messages.

Thus, there exists a need in the art for methods and compositions for detecting and differentially diagnosing conditions characterized by over-expression or inappropriate expression of WT1.

Summary of the Invention

In one aspect, the present invention provides a hybridoma cell line secreting a monoclonal antibody (MAb) specific for a protein antigen, referred to as WT1-6F [SEQ ID NO: 2], which contains amino acids 1-181 of WT1 [SEQ ID NO: 4]. One such cell line is an H2-secreting line, deposit designation ATCC No. 11598. Another cell line which is an embodiment of this aspect is the H7-secreting line, deposit designation ATCC No. 11599.

Still a third cell line is the HC17-secreting line, deposit designation ATCC No. 11600.

In another aspect, the present invention provides a monoclonal antibody produced by a cell line described
5 above. Three such antibodies, designated H2, H7 and HC17 are described herein.

In yet another aspect, the invention provides the heavy chain and light chain variable region polypeptides of the MAbs of the invention, and other fragments
10 thereof, such as Fab fragments, F(ab)₂ fragments, Fv fragments and the like.

In still another aspect, the present invention provides methods of diagnosing malignancies characterized by over-production or inappropriate expression of WT1
15 protein. These methods involve screening biological samples with antibodies of the invention, described above.

In a further aspect, the present invention provides methods of monitoring treatment of conditions
20 characterized by over-production or inappropriate expression or production of WT1 protein. One embodiment of such a method involves monitoring leukemia treatment, particularly determining the level of active leukemia following a treatment cycle.

In another aspect, the present invention provides methods for differentiating between malignancies characterized by over-production or inappropriate
25 expression of WT1 protein and conditions having similar symptomatic profiles. One embodiment of such a method involves distinguishing between mesotheliomas and
30 conditions characterized by inflammatory reactions.

In a still another aspect, the present invention provides kits useful for detecting, monitoring, and/or diagnosing a disease characterized by the expression of
35 the Wilms' tumor antigen comprising a MAb raised against

the WT-6F antigen [SEQ ID NO: 2]. Desirably, the H2, H7, HC17 MAbs or a cocktail of these, is included in such a kit.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 is a schematic diagram of the Wilms' tumor protein functional domains. The WT1 protein contains two discrete functional domains: the amino terminus contains a transcriptional regulator domain and the carboxy terminus contains a DNA binding domain with four C₂H₂ zinc fingers. G/P refers to the glutamine- and proline-rich region at the amino-terminus; ZN refers to four contiguous zinc fingers at the carboxyl-terminus. Alternatively spliced transcripts of WT1 are produced which insert 17 amino acids, VAAGSSSSVKWTEGQSN, [SEQ ID NO: 7] (17AA) within the transcriptional regulatory domain (at amino acid 249 of SEQ ID NO: 4) or a tripeptide encoding the amino acid KTS within the DNA binding domain (at amino acid 390 of SEQ ID NO: 4) between zinc fingers 3 and 4. The significance of the alternatively spliced WT1 transcripts is not known.

Fig. 2 provides the nucleic acid and amino acid sequences of the WT-6F antigen [SEQ ID NOS: 1 and 2] in which amino acids 1-11 represent a histidine fusion protein to facilitate purification; amino acids 12-192 are amino acids 1-181 of the WT1 protein; and amino acids 193-210 of SEQ ID NO: 2 are vector sequences added during cloning.

Fig. 3 provides the nucleotide and amino acid sequences of the full length WT1 protein [SEQ ID NOS: 3 and 4]. The 3' non-coding sequence of the mRNA is omitted in this figure.

Detailed Description of the Invention

The present invention provides hybridomas secreting monoclonal antibodies (MAbs) specific for epitopes found in the amino terminal amino acids 1-181 of the Wilms' tumor (WT1) protein [SEQ ID NO: 4]. The MAbs of this invention are useful in identifying, monitoring and diagnosing conditions characterized by over-expression or inappropriate expression of the WT1 protein. The MAbs do not cross-react with the ubiquitous and closely related early growth response (Egr1) family of proteins which share approximately 50% homology within the DNA binding domain located in the carboxyl terminal amino acids 275-429 of WT1 [SEQ ID NO:4]. Therefore, when used in a diagnosis based on the detection of WT1 protein, the MAbs of this invention eliminate false positives currently produced in detection methods by the use of currently available WT1 antibodies which are specific for epitopes in the zinc finger domain of the protein.

20 I. Definitions

As used herein "functional fragment" is a partial complementarity determining region (CDR) sequence or partial heavy or light chain variable sequence of an antibody which retains the same antigen binding specificity and/or neutralizing ability as the antibody from which the fragment was derived.

A "condition characterized by over-expression or inappropriate expression of WT1" refers to a cancer or other abnormal physiological state which exhibits an increased level of expression of WT1 or exhibits expression of a mutant WT1 protein, or exhibits expression of WT1 protein where such expression should normally not occur. Such increased WT1 expression has been detected in cells derived from ovarian carcinomas, mesotheliomas, prostate cancer and leukemias.

Ordinarily, in normal tissues, WT1 protein is absent or present in such low levels that it cannot be detected using conventional techniques, such as northern blot hybridization or reverse transcriptase polymerase chain reaction (RT-PCR). In contrast to WT1 protein, when a patient exhibits a "condition characterized by over-expression or inappropriate expression of WT1" as defined herein, the presence of WT1 protein can be detected using the reagents of the invention and standard techniques, e.g. immunohistochemical procedures, including immunoblotting and immunofluorescence, Western blot analysis, and enzyme-linked immunosorbant assay (ELISA). The presence of WT1 mRNA in such patients can be detected using Northern blot analysis or RNA reverse transcription PCR techniques. Background levels of WT1 can be determined by measuring such levels in the tissues where WT1 is not normally expressed (as described above) in persons not afflicted with disease.

"CDRs" are defined as the complementarity determining region amino acid sequences of an antibody. CDRs are contained within the hypervariable regions of immunoglobulin heavy and light chains. CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. CDRs of interest in this invention are derived from donor antibody variable heavy and light chain sequences, and include functional fragments and analogs of the naturally occurring CDRs, which fragments and analogs also share or retain the same antigen binding specificity and/or neutralizing ability as the donor antibody from which they were derived.

By 'sharing the antigen binding specificity or neutralizing ability' is meant, for example, that although a given MAb may be characterized by a certain level of antigen affinity, and a CDR encoded by a nucleic acid sequence of the same MAb in an appropriate

structural environment may have a lower or higher affinity, it is expected that CDRs of that MAb in such environments will nevertheless recognize the same epitope(s) as the MAb from which they are derived.

5 A "monoclonal antibody" refers to homogenous populations of immunoglobulins which are capable of specifically binding to WT1 protein. It is understood that WT1 protein may have one or more antigenic determinants, particularly in the amino acid sequence
10 spanning amino acids 1-181 of SEQ ID NO: 4. The antibodies of the invention may be directed against one or more of these determinants. As used herein, a "cocktail" of these antibodies comprises any combination of the antibodies of the invention.

15 A "chimeric antibody" refers to a type of engineered or recombinantly produced antibody which contains naturally-occurring variable region light chain and heavy chains (both CDR and framework regions) derived from a non-human donor antibody, such as the MAbs described
20 herein, in association with light and heavy chain constant regions derived from a human (or other heterologous animal) acceptor antibody.

 A "humanized antibody" refers to a recombinantly produced antibody having its CDRs and/or other portions
25 of its light and/or heavy variable domain framework regions derived from a non-human donor immunoglobulin, such as the MAbs of the present invention, the remaining immunoglobulin-derived parts of the molecule being derived from one or more human immunoglobulins. Such
30 antibodies can also include a humanized heavy chain associated with a donor or acceptor unmodified light chain or a chimeric light chain, or vice versa.

 A "bi-specific antibody" refers to an antibody derived from the Fab portions of two parent antibodies,
35 each of which binds a separate antigen. The bi-specific

antibody is characterized by the ability to bind to two antigens, particularly, the antigens to which the parent antibodies bound.

5 A Fab fragment refers to a polypeptide containing one entire light chain and amino terminal portion of one heavy chain from an antibody, such as the MAbs of this invention. A F(ab')₂ fragment refers to the fragment formed by two Fab fragments bound by disulfide bonds.

10 II. Hybridoma Cell Lines and MAbs of the Invention

The hybridoma cell lines and monoclonal antibodies of the invention are produced by employing as antigen, a novel WT1-derived protein antigen, which contains only the N-terminal sequence of the WT1 protein. Desirably,
15 the invention employs as an immunogen a WT1 containing protein antigen, referred to as WT1-6F [SEQ ID NO: 2], which contains amino acids 1-181 of the N-terminus of the native human WT1 sequence (see Fig. 1 and SEQ ID NO: 4). This antigen has been developed by the inventors and does
20 not contain any of the zinc-finger region characteristic of the carboxyl terminal portion of the WT1 protein or any of the 17 amino acid insert of the splice variant of the protein (see Fig. 1). Additional details relating to the preparation and expression of the 6F antigen are
25 provided in Example 1 below.

Generally, the hybridoma process involves generating a B-lymphocyte to the selected antigen, which B lymphocyte produces a desired antibody. Techniques for obtaining the appropriate lymphocytes from mammals
30 injected with the target antigen, WT1-6F, are well known. Generally, the peripheral blood lymphocytes (PBLs) are used if cells of human origin are desired. If non-human sources are desired, spleen cells or lymph nodes from other mammalian sources are used. A host animal, e.g. a
35 mouse, is injected with repeated doses of the purified

antigen, and the mammal is permitted to generate the desired antibody producing cells.

Thereafter the B-lymphocytes are harvested for fusion with the immortalizing cell line. Immortalizing
5 cell lines are usually transformed mammalian cells, particularly cells of rodent, bovine and human origin. Most frequently, rat or mouse myeloma cells are employed. Techniques for fusion are also well known in the art and generally involve mixing the cells with a fusing agents,
10 e.g. polyethylene glycol.

Immortalized hybridoma cell lines are selected by standard procedures, such as HAT selection. From among these hybridomas, those secreting the desired monoclonal antibody are selected by assaying the culture medium by
15 standard immunoassays, such as Western blotting, ELISA, or RIA. Antibodies are recovered from the medium using standard purification techniques. See, generally, Sambrook et al, Molecular Cloning: A Laboratory Manual, 2nd edit., Cold Spring Harbor Laboratory, Cold Spring
20 Harbor, NY (1989). Alternatively, non-fusion techniques for generating an immortal antibody-producing hybridoma cell line may be employed to generate a hybridoma antibody, where possible, e.g. virally induced transformation.

25 The invention provides three exemplary hybridoma cell lines and the MAbs secreted therefrom produced using WT1-6F as the antigen. See Examples 2 and 3 below. These three hybridomas secrete antibodies termed H2, H7 and HC17, respectively. Each hybridoma was deposited
30 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A. ("ATCC") on March 31, 1994, pursuant to the provisions of the Budapest Treaty. The H2-secreting hybridoma was granted accession number ATCC 11598, the H7-secreting hybridoma was granted

accession number ATCC 11599, and the HC17-secreting hybridoma was granted accession number ATCC 11600.

The H2, H7 and HC17 antibodies are murine IgG₁ antibodies, and have been demonstrated to specifically
5 bind WT1 protein and not to cross-react with the closely-related Egr1 family of proteins. All three monoclonal antibodies recognize the recombinant protein in ELISA assays, and full length WT1 protein in immuno-precipitation and Western blot analysis. Preliminary
10 analysis suggests that at least two distinct epitopes in the WT1-6F protein are recognized by the three MABs. The MABs of this invention are characterized in more detail in Example 4 below.

A Western blot analysis was performed to test the
15 ability of the three MABs to detect two recombinant proteins: 6F [SEQ ID NO: 2] which contains WT1 amino acids 1-181 of SEQ ID NO: 4, and WT91 which contains WT1 amino acids 85-173 of SEQ ID NO: 4. All three MABs detect the 6F recombinant protein [SEQ ID NO: 2]
20 containing WT1 amino acids 1-181. However, only H2 and H7 detect the WT91 recombinant protein containing amino acids 85-173 of WT1, suggesting that H2 and H7 recognize an epitope within the WT1 amino acid sequence 85-173 and HC17 recognizes an epitope outside this region.

25 These MABs are useful as diagnostic reagents, and possibly as therapeutic reagents as described in more detail below.

III. MAB Antibody Fragments

30 The present invention also includes functional fragments of the MABs defined above, preferably those derived from the H2, H7 and/or HC17 MABs of the invention. Such functional fragments include the heavy chain and light chain variable region polypeptides of the

MABs of the invention, and other fragments thereof, such as Fab fragments, F(ab)₂ fragments, Fv fragments and the like.

These fragments are useful as diagnostic reagents and as donors of sequences, including the variable regions and CDR sequences, useful in the formation of recombinant, chimeric, humanized or bi-specific antibodies. Techniques for generating such antibodies and antibody fragments are known in the art. For example, the functional fragments of the invention may be obtained using conventional genetic engineering techniques. See, generally, Sambrook et al., cited above. Alternatively, desired portions thereof, e.g. the CDR sequences, may be chemically synthesized.

These antibody functional fragments are useful in the assays of the invention to diagnose WT1 over-expression or inappropriate expression in specific tumors, which assays are described in more detail below. For example, by conjugating these antibody fragments to enzymes, such as horseradish peroxidase, these fragments may be employed in a conventional one-step detection assay.

IV. Diagnostic Reagents and Kits

The invention includes kits of reagents for use in immunoassays, particularly sandwich immunoassays. Such kits include a solid phase support, a monoclonal antibody of the invention, a functional fragment thereof, or a cocktail thereof, and means for signal generation. The antibodies of the invention may be pre-attached to the solid support, or may be applied to the surface of the solid support when the kit is used. The signal generating means may come pre-associated with an antibody of the invention or may require combination with one or more components, e.g. buffers, antibody-enzyme

conjugates, enzyme substrates, or the like, prior to use. Kits may also include additional reagents, e.g. blocking reagents for reducing nonspecific binding to the solid phase surface, washing reagents, enzyme substrates, and
5 the like. The solid phase surface may be in the form of microtiter plates, microspheres, or other materials suitable for immobilizing proteins. Preferably, an enzyme which catalyzes the formation of a chemiluminescent or colored product is a component of the
10 signal generating means. Such enzymes are well known in the art.

Such kits are useful in the detection, monitoring and diagnosis of conditions characterized by over-expression or inappropriate expression of the WT1
15 protein.

V. Diagnostic Assays

The MABs, fragments, reagents and kits of the invention may be used with standard assay formats for the
20 identification and diagnosis of conditions characterized by WT1 expression, over-expression or inappropriate expression, particularly in tumor/leukemic cells. The detection and measurement of WT1 expression in tissue that does not normally express WT1 or over-expression or
25 inappropriate expression in tissue that does normally express WT1 may be accomplished by resort to several known techniques, e.g., immunofluorescence (detection of WT1 protein in fixed cells/tissues) and detection of WT1 protein of whole cell extracts by western analysis. Most
30 particularly, the MABs and other compositions of this invention may be used to detect WT1 expression in abnormal kidney and genitourinary development and cancers which over-express WT1, particularly, leukemias, mesothelioma, granulosa, prostate and ovarian cancers.

The reagents of the invention may also be used to monitor therapy of such conditions.

Desirably, the MABs and fragments thereof, when used as diagnostic reagents are conventionally labelled for use as molecular weight markers or for use in ELISAs, immunofluorescence, and other conventional assay formats for the measurement of WT1 in an appropriate biological sample. Suitable label systems are well known to those of skill in the art and include fluorescent compounds, radioactive compounds or elements, and a variety of enzyme systems. As used herein, suitable samples include, without limitation, whole blood, serum, plasma, tissue samples, bone marrow, and urine.

Advantageously, the MABs of the invention can be used to screen for the WT1 protein using standard antibody staining techniques, e.g. the avidin-biotin system, immunofluorescence, or the like. For example, a tissue, e.g. from a biopsy, is fixed on a slide using standard techniques. A selected MAB (or fragment thereof) of the invention is then applied to the slide and incubated under standard conditions, e.g. at room temperature for about 1 hour. A labelled anti-mouse antibody is then used for detection. Parallel experiments with positive and negative controls (minus MAB of invention) are performed. Parallel tumor tissue with MAB recognition by fixation of the paraformaldehyde and, preferably, methanol, these antibodies may be useful on routine pathology slides.

For example, the ability of these monoclonal antibodies to detect prostate cancer cells has been demonstrated. Preliminary data has demonstrated that cocktails of these antibodies, e.g., H2/HC17 and H7/HC17, are particularly well suited for this purpose.

The MABs, or functional fragments thereof, of the invention are useful in the detection of a condition characterized by over-expression of WT1 antigen, including leukemias, mesothelioma, and granulosa, or to
5 differentiate such a condition from other conditions which exhibit similar clinical symptoms. For example, a Mab of the invention can differentiate a mesothelioma from other pleural tumors; such a use is clinically significant in view of the different prognoses for
10 pleural tumors of non-adenocarcinoma origin and adenocarcinomas. Such a method involves obtaining a suitable biological sample from a patient, incubating the sample in the presence of a Mab or functional fragment thereof of the invention, and detecting the presence of
15 binding of the Mab or fragment to the biological sample. The presence of binding above background levels detected in a non-WT1 expressing normal tissue sample indicates the presence of a mesothelioma. Any tissue or established cell line which does not express WT1 mRNA may
20 serve as a standard for negative expression of WT1 protein, including those described above in the background.

Alternatively, the Mabs and fragments thereof of the invention are useful to monitor a course of treatment for
25 a condition characterized by over-expression or inappropriate expression of the WT1 antigen. For example, active leukemia (e.g. in blast crisis) cells express WT1, while inactive leukemic cells do not express WT1. Thus, during or following a treatment cycle, a
30 biological sample from the leukemia patient is periodically tested in an assay of the invention to monitor residual leukemic disease. The lack of, or reduction of levels of, binding of a Mab or fragment of the invention to the sample indicates that the course of
35 treatment, e.g., chemotherapy, is successful in reducing

the tumor or cancer. Similarly, the MAbs and fragments of the invention may be used to detect leukemic blast cells in purged or unpurged hematopoietic stem cell preparations intended for use in bone marrow
5 transplantation.

It is anticipated that one of skill in the art of diagnostic assays may devise other series of steps utilizing the Mabs or fragments of this invention to accomplish the detection of levels of WT1 expression
10 indicative of disease. Such assay formats are known within the art, and are simply a matter of selection. This invention is not limited by the particular assay format or assay steps employed in the diagnosis of inappropriate expression of WT1 protein in biological
15 samples.

Because the Mabs H2, H7, and HC17 were raised to a region of the WT1 amino acid sequence that is unique to the amino terminal portion of WT1 and does not contain the zinc finger DNA binding domains, these Mabs and
20 fragments have little potential for crossreactivity with non-WT1 proteins, unlike known other WT1 polyclonal and monoclonal Mabs. For example, these Mabs do not cross-react with the Egr family of proteins. Thus they permit an unambiguous positive detection of WT1 expression in
25 biological samples.

The advantages of using these Mabs for such diagnosis in comparison to the use of the known monoclonal and polyclonal antibodies of the art rely in the defined specificity of the Mabs for the amino
30 terminal sequence of WT1, their uniform binding affinity and their lack of cross-reactivity as described above.

V. Therapeutic Use of Mabs of this Invention

Further, if these Mabs of the invention are have the ability to internalize into the nucleus of the cell which expresses WT1 [see, e.g., United States Patent No. 5,296,348, issued March 22, 1994, incorporated by reference herein], they may also be employed in the treatment of such WT-1 expressing tumors or cancers. For example, these Mabs, other antibody types such as chimeric or humanized antibodies, or fragments which share the binding affinity or specificity of the whole Mab may be used to deliver toxins or therapeutic agents to the tumor or metastasis sites.

These Mabs, other antibodies and fragments of the present invention may also be employed in other therapeutic methods known to those of skill in the art.

The following examples illustrate the characterization and uses of the antibodies of the invention. These examples are illustrative only and do not limit the scope of the invention.

20

Example 1 - Preparation of the WT1-6F Antigen

A. Cloning Strategy

A recombinant protein containing the first 181 amino acids of the human WT1 was produced to use as an antigen in the preparation of WT1 specific antibodies as follows.

The amino terminus of WT1 was subcloned from 7Zf+WT1, a synthetic full-length human WT1 nucleotide sequence described in Morris et al, cited above. Briefly, the nucleotide sequence encoding the full-length protein was constructed from the partial human WT1 cDNA clone WT33 [Call et al, Cell, 60:509-520 (1990)]. The WT1 amino acids 1-84 plus an overlapping 20 amino acid segment were synthesized using overlap-extension

polymerase chain reaction. The nucleotides specifying amino acid codons were optimized for expression in *E. coli*.

The resulting synthetic DNA fragment (320 bp) was fused to the 5' end of WT33 at a unique Bst XI site centered at position WT1 amino acid 101 of SEQ ID NO: 4 (nucleotide 50 of WT33). A Cla I-Eco RI fragment was subcloned into pGem7Zf+ (Promega, Madison, WI) to produce 7Zf+WT1. From this plasmid, a Nco I-Bam HI fragment was isolated and subcloned into a modified pet11d vector (Novagen, Madison, WI).

The pet11d vector was modified by digesting with Nco I and ligating to synthetic, double-stranded oligonucleotides which contained the following 5' overhangs complementary to a Nco I restriction site to produce 6XHISpet11d:

5'-CATGAGAGGATCGCATCACCATCACCATCACTC 3'[SEQ ID NO: 5]
3' TCTCCTAGCGTAGTGCTAGTGCTAGTGAGGTAC-5'[SEQ ID NO: 6].

The synthetic oligonucleotide introduces nucleotide codons that encode the amino acids MRSHHHHHH of SEQ ID NO: 2 to produce an in-frame 5' hexa-histidine fusion protein that maintains a single Nco I restriction site at the 3' end of the sequence. The 5' hexa-histidine encoding region facilitates protein purification [Abate et al, Proc. Natl. Acad. Sci. USA, 87:1032 (1990)].

The Nco I-Bam HI fragment of 7Zf+WT1 containing the amino terminus of WT1 was subcloned into 6XHISpet11d digested with Nco-I and Bam HI to create pet11d-6F.

B. Expression in *E. coli* and Purification

The bacterial strain BL21 (Novagen, Madison, WI) was transformed with the pet11d-6F DNA. Protein expression was induced in a logarithmically growing bacterial culture with 1 mM isopropyl- β -thiogalactopyranoside (IPTG) for two to three hours at 37°C.

Bacteria were harvested by centrifugation, lysed in 6 M guanidine-HCl, 50 mM sodium phosphate, pH 8.0 for 2 hours at room temperature or overnight at 4°C, and clarified by centrifugation at 40,000 x g for 30 minutes.

5 The histidine fusion recombinant protein WT1-6F was purified by a one step affinity binding of the hexahistidines to the nickel-chelate affinity resin NTA-agarose (Qiagen, Chatsworth, CA) using recommended procedures. The purified protein was renatured by
10 dialysis into phosphate buffered saline with 5% glycerol.

Purity of the protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as follows. The purified protein was renatured by dialysis into PBS containing 5% glycerol and analyzed on
15 a 10% SDS polyacrylamide gel. Proteins were stained with Coomassie blue. The 6F recombinant protein was shown to be homogenous, migrating under denaturing conditions as a 28 kDa protein.

C. The WT1-6F Antigen

20 The 6F amino acid sequence is encoded by a synthetic nucleotide sequence shown in Fig. 2 [SEQ ID NOS: 2 and 1]. The 6F nucleotide sequence was derived from the synthetic full-length human WT1 sequence [Morris et al, cited above and SEQ ID NOS: 3 and 4]. As
25 illustrated in Fig. 2, the recombinant 6F antigen contains amino acids 1-181 of the human WT1 sequence [SEQ ID NO:4] as well as amino acids at both the amino and carboxyl ends, which sequences were introduced during cloning. The entire 6F amino acid sequence is shown in
30 Fig. 2 [SEQ ID NO: 2]. Amino acids 1-11 (MRGSHHHHHHS) of SEQ ID NO: 2 were added to produce a histidine fusion protein to facilitate purification of the recombinant protein. Amino acids 193-210 of SEQ ID NO: 2 are not WT1 sequences, but were added from the vector during cloning.
35 Note that nucleotides 1-333 [SEQ ID NO: 1] are synthetic

sequences optimized for protein translation in *E. coli*; the remaining nucleotides are derived from the human WT33 cDNA clone. This does not change the human WT1 amino acid sequence, but increases efficiency of protein expression in *E. coli* [Rauscher et al, Science, 250:1259-1262 (1990), Abate et al, Proc. Natl. Acad. Sci., 87:1032-1036 (Feb. 1990)].

A second recombinant protein, WT91 (described in Morris et al, cited above) contains the amino acids 85-173 of SEQ ID NO: 4.

Example 2 - Preparation of Antisera and Immunization

Rabbit polyclonal antisera was produced by CoCalico Biologicals, Inc. Rabbits were immunized subcutaneously with 100 µg of 6F recombinant protein of Example 1 and boosted at two to three week intervals. The rabbit sera was used without further purification.

Example 3 - Preparation of Monoclonal Antibodies

Fifty micrograms of purified recombinant protein of Example 1 was injected subcutaneously into the hind footpads of BALB/c mice every two weeks for a total of three injections. Sera was collected from the tail, and tested for WT1 specific antibodies by immunoprecipitation of 35S-methionine labeled *in vitro* translated human WT1 protein.

Two weeks later, 50 µg of protein in 200 µl of saline was injected intravenously followed by removal of each animal's spleen. Spleen cells were fused with a myeloma cell line, P3X63AG8/653 [ATCC CRL 1580], using standard techniques.

The resulting hybridomas producing MAbs H2, H7 and HC17 were screened in a two step process. Positive clones were initially identified using an enzyme-linked immunosorbent assay (ELISA) against the 6F recombinant

protein. Secondary screening was carried out using immunoprecipitation of full length WT1 protein produced by *in vitro* translation (IVT). These experiments demonstrated that the MAbs H2, H7 and HC17 specifically
5 recognize the WT1 protein and that they appear to recognize distinct epitopes on the WT1 protein.

1. Immunoprecipitation

Full length WT1 was expressed *in vitro* from by transcribing RNA from Eco RI linearized vector 7Zf+WT1
10 with SP6 RNA polymerase, and translating protein in rabbit reticulocyte lysate with ³⁵S-methionine. The ³⁵S-methionine labeled protein is 55 kDa and is specifically immunoprecipitated by rabbit polyclonal anti-6F sera, and by the mouse monoclonal antibodies H2, H7, and HC17.

15 Immunoprecipitations were done as previously described in Morris et al, cited above. Briefly, IVT WT1 was added to radioimmunoprecipitation buffer with protease inhibitors (RIPA: 10 mM Tris-Cl pH 7.4, 150 mM sodium chloride, 1 mM ethylenediamine-tetraacetic acid
20 (EDTA), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 2 µg/ml leupeptin and 2 µg/ml aprotinin) along with antibodies and incubated 90 minutes at 4°C. Either 30 µl of 10% *Staphylococcus* A (Pansorbin, Calbiochem, San Diego, CA)
25 or 100 µl of 50% Protein A Sepharose (Pharmacia, Piscataway, NJ) was added and incubated for 15 minutes (Staph A) or 30 minutes (Protein A). The immune complexes were collected by centrifugation in the microfuge and washed with 0.5-1.0 ml of RIPA 3 to 4
30 times. The immunoprecipitated proteins were analyzed on 10 or 15% SDS-polyacrylamide gels and visualized by autoradiography.

The resulting SDS PAGE gel demonstrated that MAbs of this invention immunoprecipitate WT1 expressed by
35 *in vitro* transcription and translation.

2. Baculovirus expression of full length WT1

The full length WT1 protein encoding sequence was subcloned from 7Zf+WT1 into a baculovirus expression vector. Sf9 insect cells were infected with WT1-
5 baculovirus and cells harvested 48-96 hours following infection. Cells were pelleted by centrifugation, washed three times in PBS. Whole cell lysates were prepared by lysing a cell pellet in 10 times the cell pellet volume with Laemmli loading buffer (50 mM Tris-Cl, pH 6.8, 100
10 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol).

Ten μ l of WT1 lysate were analyzed on a 10% SDS-polyacrylamide gel. Western analysis of protein was performed as follows. A whole cell lysate of Sf9 cells
15 expressing baculovirus encoded WT1 protein was separated on a 10% SDS-polyacrylamide gel and transferred to 0.45 μ m BA 85 nitrocellulose (Schleicher and Schuell, Keene, NH) using semi-dry electroblot transfer for 60-90 minutes at 4 mAmps/cm². Molecular weight standards were cut from
20 the blot and stained with Amido black and the nitrocellulose filter blot was blocked in 5% BSA-PBS for 60 minutes at room temperature or overnight at 4°C. The primary antibody was diluted in blocking buffer (rabbit anti-6F 1:400; the monoclonal antibodies of the invention
25 1:500 or 1:1000) and added to filters for 30 to 60 minutes at room temperature.

Filters were rinsed briefly twice in wash buffer (PBS, 0.1% BSA, 1% Tween 20) and three times for 10 minutes each while shaking vigorously. Soluble
30 protein A conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) was diluted 1:5000 in 5% BSA-PBS and incubated for 30 minutes at room temperature. Filters were washed as before, rinsed in PBS, and incubated with a 1:1 mixture of the ECL substrates A and
35 B (Amersham, Arlington Heights, IL) for 1 minute at room

temperature. Filters were removed from the liquid, excess moisture drained, and wrapped in Saran wrap and immediately exposed to film (average exposure 15 seconds to 3 minutes).

5 The gels revealed that the polyclonal and monoclonal antibodies of this invention specifically detect a 55 kDa protein in Sf9 cells transfected with WT1 baculovirus expression vector and not cells mock transfected.

10

Example 4 - Characterization of Murine MAbs H2, H7 and HC17

15 To determine whether the WT1 monoclonal antibodies of the present invention detect different epitopes within the first 181 amino acid of the 6F antigen, purified recombinant proteins 6F (WT1 amino acid 1-181) and WT91 (WT1 amino acid 85-173) were separated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. Western blot analysis was performed as described in

20 Example 3.

 Polyclonal antibodies were diluted 1:400 and monoclonal antibodies diluted 1:500. The polyclonal antisera recognizes both the 6F and WT91 recombinant proteins. The monoclonal antibodies H2 and H7 recognize

25 both 6F and WT91 recombinant proteins, suggesting they detect an epitope with amino acid 85-173 of WT1 [SEQ ID NO:4]. HC17 does not detect the WT91 recombinant protein indicating that it recognizes an epitope outside of this

30

35

Example 5 - Detection of WT1 Protein in Human Acute Leukemias

The following study demonstrates that a MAb of the invention, H2, is able to distinguish between leukemic blast cells and normal mononuclear cells by detecting the WT1 protein in nuclei of leukemic blast cells. No WT1 protein was detected in the nuclei of normal mononuclear cells or mononuclear cells by either immunofluorescence or by conventional reverse-transcriptase polymerase chain reaction (RT-PCR) techniques.

A. Samples

Mononuclear cell preparations of 110 adult leukemia patients were examined in this study, T-cell acute lymphoblastic leukemias (T-ALL) had been diagnosed in 27, common acute lymphoblastic leukemias (c-ALL) in 28, pre-pre-B cell acute lymphoblastic leukemias (ppB-ALL) in 8, acute myelogenous leukemias (AML) in 40, chronic myelogenous leukemias in blast crisis (one lymphatic and three myeloid CML-BC) in 4 and chronic myelogenous leukemias in chronic phase (CML-CP) in 3 patients. Controls were 4 patients with reactive bone marrow aspirates who had fever of unknown origin (H.M., G.S.), anemia secondary to iron deficiency (V.H.) or limited-disease esophageal cancer with no morphological evidence of bone marrow infiltration (H.F.).

Mononuclear cells were isolated from bone marrow aspirates or in a few cases from peripheral blood samples by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Freiburg, Germany). Also, peripheral mononuclear cells enriched with CD34⁺ hematopoietic progenitors were obtained from five patients (S.K., S.Kt., K.D., N.G., H.G.) who had solid cancer with no morphological evidence of bone marrow infiltration. Their mononuclear cells had been harvested by leukapheresis during the recovery phase following a

course of progenitor-cell-mobilizing chemotherapy and G-CSF. Furthermore, a 91% pure peripheral CD34⁺ hematopoietic progenitor cell suspension was prepared from the leukapheresis product of a patient (G.M.) suffering from plasmacytoma.

The number of peripheral CD34⁺ progenitors was determined using a FACScan cytofluorometer. At least 10⁵ CD34⁺ vital cells per sample were available for testing. In addition, nucleated blood cells of twenty patients with non-neoplastic disease were isolated using a red blood cell lysis-buffer (150 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA). The leukemia cell line K562 [ATCC CCL 243] served as the positive control in detection of wt1 mRNA and in immunofluorescence studies.

B. Indirect Immunofluorescence Assay

For the indirect immunofluorescence assay, mononuclear cells of bone marrow were isolated as already described. In addition, a 91% pure CD34⁺ hematopoietic progenitor cell suspension was prepared from the leukapheresis product of a patient (G.M.) suffering from plasmacytoma. Prior to leukapheresis, she underwent peripheral stem-cell mobilization with chemotherapy (VAD-protocol) and G-CSF.

An aliquot taken from the leukapheresis product contained 2.5 x 10⁸ mononuclear cells and, according to FACS analysis [M. Notter et al, Blood, 82:3113 (1993)], 8.75 x 10⁶ CD34⁺ hematopoietic progenitor cells. First, T-lymphocytes and myeloid cells were depleted using paramagnetic microbeads coupled with mouse anti-human CD3 and CD33 MAbs (Miltenyi, Cologne, Germany). Using a B2 column (Miltenyi), the cells were sorted according to the manufacturer's instructions. Subsequently, CD34⁺ hematopoietic progenitor cells were isolated using the CD34 Progenitor Isolation Kit (QBEND/10) from Miltenyi. After removal of unbound MAb by washing, cells were

passed twice over a Mini MACS column (without flow resistor, Miltenyi). The 8G12-PE MAb (Becton Dickinson, Heidelberg, Germany) was used to determine the purity of the final CD34⁺ cell suspension, which was 91% with a
5 yield of 39%.

One fraction of the cell preparations was processed according to the RT-PCR protocol described to detect the wt1 transcript. Another fraction was used in the immunofluorescence assay. K562 cells served as
10 positive controls. For detection of the nuclear protein WT1, 5 x 10⁴ mononuclear cells were cytocentrifuged onto glass slides and air-dried for 2 hours. To destroy cellular membranes, the cells were exposed to pure methanol for 30 minutes at 4°C and then washed twice in
15 PBS. The cells were incubated for 45 minutes at 4°C with the mouse antihuman WT1 MAb H2, produced as described in Example 3 above, or a negative control MAb (MAb 425) recognizing the EGF-receptor [Rodeck et al, Cancer Res., 47:3692 (1987)]. The cells were washed again in PBS and
20 incubated for 30 minutes with fluorescein isothiocyanate (FITC)-conjugated goat antimouse F(ab')₂ fragments (Immunotech, Marseille, France). After washing in PBS, cells were embedded in PBS-glycerin and analyzed by fluorescence microscopy (Axiophot, 1000x, Zeiss,
25 Oberkochen, Germany). Results are reported below in Table 1.

Table 1

	<u>Diagnosis</u>	<u>Patient Initials</u>	<u>wt1 mRNA Expression</u>	<u>Nuclear Immunofluorescence</u>	
				<u>Mab H2</u>	<u>Mab 425</u>
5	<u>ALL</u>				
	pre-pre-B-ALL	C.R.	yes	#yes	\$no
	c-ALL	R.P.	yes	yes	no
	c-ALL	F.G.	yes	yes	no
	c-ALL	W.T.	no	no	no
10	T-ALL	A.D.	yes	no	no
	T-ALL	M.S.	no	no	no
	<u>AML</u>				
	AML-FAB-M2	M.E.	yes	yes	no
15	AML-FAB-M4	A.M.	yes	yes	no
	AML-FAB-M2	H.K.	yes	no	no
	AML-FAB-M1	H.L.	no	no	no
	<u>Controls</u>				
20	K562 cells		yes	yes	no
	CD34+91% pure progenitor cells G.M.		no	no	no
25	normal blood mononuclear cells		no	no	no
	# indicates more than 30% of cells show a strong nuclear fluorescence.				
30	\$ indicates no cells show nuclear fluorescence.				

The indirect immunofluorescence assay with the Mab H2 directed against the WT1 nuclear protein disclosed
 35 a strong and specific nuclear fluorescence in blast cells from 3 of 6 ALL patients and 2 of 4 AML patients tested (Table 1). No nuclear immunofluorescence was observed in 3 ALL patients, one with (A.D.) and two without wt1 gene expression. In mononuclear cell preparations from 4 AML
 40 patients a nuclear immunofluorescence with Mab H2 was found in 2 cases and both tested positive for wt1 mRNA expression using RT-PCR. While blast cells of one AML patient did not express the wt1 mRNA and had no nuclear immunofluorescence with Mab H2, those of another AML
 45 patient did show transcription of the wt1 mRNA but no nuclear immunofluorescence (H.K., Table 1). K562 cells

showed strong nuclear immunofluorescence with MAb 6F-H2, whereas normal mononuclear blood cells and cells of a 91% pure CD34⁺ hematopoietic progenitor cell suspension did not (Table 1). There was no nuclear immunofluorescence detectable using the negative control MAb 425 (Table 1). In normal blood granulocytes, cytoplasmic but no nuclear fluorescence was found with MAb H2 and regarded as unspecific (data not shown).

Immunofluorescence using MAb H2 confirms RT-PCR data, and shows detection of the WT1 protein in nuclei of leukemic blast cells but not in those of normal mononuclear cells or mononuclear cells enriched with CD34⁺ hematopoietic progenitors.

Expression of protein occurs following the transcription of mRNA message from the double stranded DNA. This mRNA is translated into a protein. Detectable mRNA indicates that the necessary "intermediate" is present and potentially capable of being translated into protein. However, this correlation does not always occur and the presence of mRNA does not necessarily mean the protein is being produced. Therefore, immunofluorescence detects protein expression and is the preferable assay system.

Example 6 - Detection of WT1 Protein in Malignant Mesotheliomas

A. Cell Lines

The mesothelioma cell lines (ML1-ML19) used in the study were all developed from human mesothelioma tumors diagnosed using conventional immunohistochemical tests. Cell lines ML-10 and ML-16 were established by explant culture at the University of Pennsylvania [W. R. Smythe et al, Ann. Thorac. Surg., 57(6):1395-1401 (1994)]. Both cell lines have been passaged over 25 times without evidence of senescence, grow as tumors in

immunodeficient mice, and show a staining pattern characteristic of mesothelioma with lack of staining with LeuM1 and carcinoembryonic antigen (CEA) antibodies. Cell lines ML1-ML8 were developed in the Surgical
5 Oncology Laboratory at the National Cancer Institute (USA). Mesothelioma cell lines, (ML11-ML15) and lung cancer lines (LL5-LL8) were provided by Dr. Carmen Allegra from the Medical Oncology Branch, NCI-Navy, National Naval Medical Center. Cell lines: ML9 (H-Meso),
10 ML17, ML18 and ML19 were provided by Dr. Joseph Testa from Fox Chase Cancer Institute, Philadelphia, PA. Normal mesothelial cells were developed from explants derived from non-malignant visceral pleural tissue obtained at surgery.
15 These cell lines were maintained in RPMI-40 media (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal calf bovine serum, non-essential amino acids (10 mM), L-Glutamine (200 mM), penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml). The six lung cancer cell
20 lines, LL1 (A549), LL2 (Calu-1), LL3 (Calu-3), LL4 (Calu-6), LL9 (SK-LU-1), LL10 (SK-MES-1), were purchased from American Type Culture Collection (ATCC) and cultured per instructions. Normal bronchial epithelial cells [S. A. Mette et al, Am. J. Respir. Cell. Mol. Biol., 8:562-572
25 (1993)] (HBE4) and human umbilical vein endothelial cells were cultured as described in S. M. Albelda et al, J. Clin. Invest., 83:1992-2002 (1989)].

B. Transfection Protocol

To generate a positive control for cellular
30 localization studies of WT1 protein, COS-1 cells (ATCC) were either seeded at 5×10^4 cells/cm² onto 1% gelatin-coated coverslips or at 5×10^5 cells in a 35 mm dish and maintained in DMEM (Gibco-BRL, Gaithersburg, MD) plus 10% fetal bovine serum. Twenty-four hours later, 2.5 µg of
35 pCMVhuWT1cDNA, an expression vector described previously

[Morris et al, cited above] was transfected into the cells by the calcium phosphate-mediated co-precipitation method [J. Sambrook et al, cited above]. Three days later the cells on the coverslips were processed for immunofluorescence staining with WT1 antibody and cells in 35 mm dish were harvested for immunoblot analysis which is described below.

C. Human Tissue and Tumor Specimens

Excess tissue specimens from normal organs, 9 mesothelioma tumors (Table 2), and 9 non-small cell lung carcinomas (NSCLC) were obtained freshly at the time of surgery and either immediately frozen in liquid nitrogen or frozen on dry ice after embedding in O.C.T. compound (Miles Scientific, Elkhart, IN). Samples were stored at -70°C until further analysis. All diagnoses for the tumors were made by the pathologists at the University of Pennsylvania based on conventional histological and clinical criteria. Mesothelioma tumors were stained immunohistochemically and were characteristically negative for LeuM1 and CEA. Results are reported in Table 2 below.

Table 2

<u>Sample</u>	<u>Age</u>	<u>Sex</u>	<u>Histologic Type</u>
MT1	56	M	Epithelial malignant mesothelioma (MM)
25 MT2	69	F	Epithelial MM
MT3	59	F	Mixed MM
MT4	51	M	Spindle Cell MM
MT5	61	M	Mixed MM
MT6	72	M	Fibrosarcomatous MM
30 MT7	70	M	Inflammatory MM
MT8	65	M	Epithelial MM
MT9	-	0	Benign fibrous tumor

D. Immunoblot Analysis

To determine if the WT1 protein was expressed in mesothelioma cell lines, immunoblotting experiments were performed, as follows, on nuclear extracts using the
5 H2 anti-WT1 MAb prepared as described in Example 3 above.

Nuclear extracts were prepared from cell lines using standard techniques [F. M. Ausubel et al, In Current Protocols in Molecular Biology, John Wiley and Sons, New York (1991)]. The nuclear pellet was collected
10 by centrifugation at 4000 rpm for 15 minutes at 4°C, resuspended in 5 times the pellet volume in electrophoresis sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, pH 6.8), and boiled for 5 minutes. Seventy-five µl of nuclear extract was applied on a 10%
15 SDS-polyacrylamide gel under reducing conditions. The separated proteins were transferred to a nitrocellulose membrane which was developed as previously described [K. A. Knudsen et al, Exp. Cell. Res., 157:218-226 (1985)] using anti-WT1 as a primary antibody and an alkaline
20 phosphatase-coupled anti-mouse as the secondary antibody.

The H2 MAb recognized a 52 KDa protein from the COS-1 cells transfected with pCMVhuWT1cDNA. No WT1 expression was seen in non-transfected cells or in LL1, a lung cancer cell. However, in the ML17, ML13, ML16, and
25 ML14 mesothelioma cell lines the antibody recognized two (52 and 55 KDa) proteins in varying amounts.

E. Immunolocalization Studies

1. Immunofluorescence

In order to determine the cellular
30 location of the WT1 protein and to confirm the immunoblotting experiments, immunofluorescence staining was performed on some of the mesothelioma cell lines, as follows.

Cell lines ML13 and ML16 which express elevated levels of WT1 mRNA (determined using conventional RT-PCR techniques) were analyzed and LL1 used as a negative control, since it expressed almost no WT1 mRNA. An isotyped matched monoclonal antibody against the endothelial cell specific molecule, PECAM-1 was used as a non-reactive control. Confluent monolayers of cells grown on glass coverslips coated with 1% gelatin were processed as previously described [S. M. Albelda et al, cited above]. Immunofluorescence studies were performed with a 1:250 dilution of anti-WT1 ascites and a 1/200 fluorescein-conjugated anti-mouse antibody (Cappel Labs, Malvern, PA). The coverslips were evaluated under epifluorescence. COS-1 cells grown on coverslips and transfected with pCMVhuWT1cDNA, were used as a positive control.

COS-1 cells transfected with pCMVhuWT1cDNA stained strongly with the monoclonal anti-WT1 H2 with expression confined to the nucleus. In contrast, the untransfected COS-1 cells showed only baseline fluorescence. A similar nuclear staining pattern has been seen in COS-1 cells transfected with WT1cDNA and stained with a polyclonal anti-WT1 antibody [J. F. Tet al, cited above]. Clear nuclear staining with the anti-WT1 H2 MAb was also seen in the ML13 and the ML16 mesothelioma cell lines. In contrast, the lung cancer cell line (LL1) which did not express any WT1 mRNA did not stain positively with the anti-WT1 antibody. No appreciable staining was seen with the control antibody on any of the cell lines tested indicating the specificity of WT1 MAb.

2. Immunohistochemistry

In addition to evaluating WT1 protein expression in cell lines, the WT1 protein expression was evaluated in tissues by immunohistochemical staining.

Frozen sections from 5 mesotheliomas and 5 NSCLC solid tumor specimens were stained with anti-WT1 MAb and a control MAb.

For immunohistochemistry, thin sections (5 μ m) were prepared from frozen tissues embedded in O.C.T., fixed in acetone at -20°C for 5 minutes and stored at -70°C. Prior to staining, the sections were blocked with 5% horse serum in PBS and washed twice in PBS at room temperature. The sections were incubated with a 1/1000 dilution of primary WT1 monoclonal antibody diluted in PBS/4% bovine serum albumin (BSA) for 1 hour at room temperature. Sections were washed twice in PBS/4% BSA, and then incubated for 30 minutes with a 1/1000 diluted biotinylated IgG horse antibody to mouse. The streptavidin-biotin ABC peroxidase detection system (Vector, Burlingame, CA) was applied, followed by a 2 minute incubation with 3-amino-9-ethylcarbazole (AEC) (Zymed, San Francisco, CA) as the substrate. The sections were mounted and evaluated microscopically.

Strong, primarily nuclear, staining was noted in a subset of identifiable neoplastic cells (5-10%) in all 5 mesothelioma tumors. Nuclear staining was not observed with a control MAb. Immunohistochemical staining of WT-1 was not observed, in any of the 5 non-small cell lung carcinomas examined as illustrated for LC4 and LC8.

F. Results

Immunohistochemical staining of both the mesothelioma tumors and the cell lines with the anti-WT1 monoclonal antibody, H2, further revealed that WT1 protein is expressed abundantly. As predicted for a transcription factor, the WT1 protein localized to the nucleus in a proportion of cells in culture and in tumors. Although the staining of WT1 protein has been observed in F9 embryonic carcinoma cells and in K562

cells [A. Telerman et al, Oncogene, 8:2545-2548 (1992)], immunohistochemical localization of WT1 in human tissues has not been previously reported. The general pattern of the expression WT1 protein was somewhat heterogeneous in mesothelioma tumors, however, WT1 was consistently expressed in at least some cells of all the tumors examined. Immunoblot analysis of nuclear extracts from mesothelioma cell lines revealed the presence of a 52 KDa and a 54 KDa sized WT1 proteins. Whether the two proteins represent alternatively spliced WT1 iso-forms [D. A. Haber et al, Proc. Natl. Acad. Sci. USA, 88:9618-9622 (1991)] or a single form differently processed in the cancer cells is not known.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: The Wistar Institute of Anatomy and Biology
- (ii) TITLE OF INVENTION: WT1 Monoclonal Antibodies
and Methods of Use Therefor
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
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PO Box 457
 - (C) CITY: Spring House
 - (D) STATE: Pennsylvania
 - (E) COUNTRY: USA
 - (F) ZIP: 19477
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0,
Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: WO
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/234,783
 - (B) FILING DATE: 28-APR-1994
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Bak, Mary E.
 - (B) REGISTRATION NUMBER: 31,215
 - (C) REFERENCE/DOCKET NUMBER: WST48PCT
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 215-540-9200
 - (B) TELEFAX: 215-540-5818

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 633 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..630

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Arg Gly Ser His His His His His Ser Met Gly	
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TCC GAC GTT CGT GAC CTG AAC GCA CTG CTG CCG GCA GTT	78
Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val	
15 20 25	
CCG TCC CTG GGT GGT GGT GGT GGT TGC GCA CTG CCG GTT	117
Pro Ser Leu Gly Gly Gly Gly Gly Cys Ala Leu Pro Val	
30 35	
AGC GGT GCA GCA CAG TGG GCT CCG GTT CTG GAC TTC GCA	156
Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe Ala	
40 45 50	
CCG CCG GGT GCA TCC GCA TAC GGT TCC CTG GGT GGT CCG	196
Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro	
55 60 65	
GCA CCG CCG CCG GCA CCG CCG CCG CCG CCG CCG CCG CCG	234
Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro	
70 75	
CCG CAC TCC TTC ATC AAA CAG GAA CCG AGC TGG GGT GGT	273
Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly	
80 85 90	
GCA GAA CCG CAC GAA GAA CAG TGC CTG AGC GCA TTC ACC	312
Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr	
95 100	
GTT CAC TTC TCC GGC CAG TTC ACT GGC ACA GCC GGA GCC	351
Val His Phe Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala	
105 110 115	

37

TGT	CGC	TAC	GGG	CCC	TTC	GGT	CCT	CCT	CCG	CCC	AGC	CAG	390
Cys	Arg	Tyr	Gly	Pro	Phe	Gly	Pro	Pro	Pro	Pro	Ser	Gln	
		120					125					130	
GCG	TCA	TCC	GGC	CAG	GCC	AGG	ATG	TTT	CCT	AAC	GCG	CCC	429
Ala	Ser	Ser	Gly	Gln	Ala	Arg	Met	Phe	Pro	Asn	Ala	Pro	
			135						140				
TAC	CTG	CCC	AGC	TGC	CTC	GAG	AGC	CAG	CCC	GCT	ATT	CGC	468
Tyr	Leu	Pro	Ser	Cys	Leu	Glu	Ser	Gln	Pro	Ala	Ile	Arg	
	145					150					155		
AAT	CAG	GGT	TAC	AGC	ACG	GTC	ACC	TTC	GAC	GGG	ACG	CCC	507
Asn	Gln	Gly	Tyr	Ser	Thr	Val	Thr	Phe	Asp	Gly	Thr	Pro	
			160					165					
AGC	TAC	GGT	CAC	ACG	CCC	TCG	CAC	CAT	GCG	GCG	CAG	TTC	546
Ser	Tyr	Gly	His	Thr	Pro	Ser	His	His	Ala	Ala	Gln	Phe	
	170				175					180			
CCC	AAC	CAC	TCA	TTC	AAG	CAT	GAG	GAT	CCG	GCT	GCT	AAC	585
Pro	Asn	His	Ser	Phe	Lys	His	Glu	Asp	Pro	Ala	Ala	Asn	
		185					190					195	
AAA	GCC	CGA	AAG	GAA	GCT	GAG	TTG	GCT	GCT	GCC	ACC	GCT	624
Lys	Ala	Arg	Lys	Glu	Ala	Glu	Leu	Ala	Ala	Ala	Thr	Ala	
				200					205				
GAG	CAA	TAA											633
Glu	Gln												
	210												

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 210 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Arg	Gly	Ser	His	His	His	His	His	His	Ser	Met	Gly	Ser
1				5					10				
Asp	Val	Arg	Asp	Leu	Asn	Ala	Leu	Leu	Pro	Ala	Val	Pro	Ser
15				20					25				
Leu	Gly	Gly	Gly	Gly	Gly	Cys	Ala	Leu	Pro	Val	Ser	Gly	Ala
	30					35					40		

38

Ala Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala
 45 50 55
 Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala
 60 65 70
 Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile Lys
 75 80
 Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln
 85 90 95
 Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr
 100 105 110
 Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro
 115 120 125
 Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro
 130 135 140
 Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala
 145 150
 Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr
 155 160 165
 Pro Ser Tyr Gly His Thr Pro Ser His His Ala Ala Gln Phe
 170 175 180
 Pro Asn His Ser Phe Lys His Glu Asp Pro Ala Ala Asn Lys
 185 190 195
 Ala Arg Lys Glu Ala Glu Leu Ala Ala Ala Thr Ala Glu Gln
 200 205 210

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1680 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 381..1670

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

GTTCAAGGCA GCGCCACAC CCGGGGGCTC TCGCAACCC GACCGCTGT 50
CCGCTCCCCC ACTTCCCGCC CTCCCTCCCA CCTACTCATT CACCCACCCA 100
CCCACCCAGA GCCGGGACGG CAGCCCAGGC GCCCGGGCCC CGCCGTCTCC 150
TCGCCGCGAT CCTGGACTTC CTCTTGCTGC AGGACCCGGC TTCCACGTGT 200
GTCCCGGAGC CGGCGTCTCA GCACACGCTC CGCTCCGGGC CTGGGTGCCT 250
ACAGCAGCCA GAGCAGCAGG GAGTCCGGGA CCCGGGCGGC ATCTGGGCCA 300
AGTTAGGCGC CGCCGAGGCC AGCGCTGAAC GTCTCCAGGG CCGGAGGAGC 350
CGCGGGGCGT CCGGGTCTGA GCCTCAGCAA ATG GGC TCC GAC GTG 395
                               Met Gly Ser Asp Val
                               1           5

CGG GAC CTG AAC GCG CTG CTG CCC GCC GTC CCC TCC CTG 434
Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro Ser Leu
                               10           15

GGT GGC GGC GGC GGC TGT GCC CTG CCT GTG AGC GGC GCG 473
Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala
                               20           25           30

GCG CAG TGG GCG CCG GTG CTG GAC TTT GCG CCC CCG GGC 512
Ala Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly
                               35           40

GCT TCG GCT TAC GGG TCG TTG GGC GGC CCC GCG CCG CCA 551
Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro
                               45           50           55

CCG GCT CCG CCG CCA CCC CCG CCG CCG CCG CCT CAC TCC 590
Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser
                               60           65           70

TTC ATC AAA CAG GAG CCG AGC TGG GGC GGC GCG GAG CCG 629
Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro
                               75           80

CAC GAG GAG CAG TGC CTG AGC GCC TTC ACT GTC CAC TTT 668
His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe
                               85           90           95

TCC GGC CAG TTC ACT GGC ACA GCC GGA GCC TGT CGC TAC 707
Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr
                               100           105

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GGG Gly 110	CCC Pro	TTC Phe	GGT Gly	CCT Pro	CCT Pro	CCG Pro	CCC Pro	AGC Ser	CAG Gln	GCG Ala	TCA Ser	TCC Ser	746
GGC Gly	CAG Gln	GCC Ala	AGG Arg	ATG Met	TTT Phe	CCT Pro	AAC Asn	GCG Ala	CCC Pro	TAC Tyr	CTG Leu	CCC Pro	785
AGC Ser	TGC Cys	CTC Leu	GAG Glu	AGC Ser	CAG Gln	CCC Pro	GCT Ala	ATT Ile	CGC Arg	AAT Asn	CAG Gln	GGT Gly	824
TAC Tyr	AGC Ser	ACG Thr	GTC Val	ACC Thr	TTC Phe	GAC Asp	GGG Gly	ACG Thr	CCC Pro	AGC Ser	TAC Tyr	GGT Gly	863
CAC His	ACG Thr	CCC Pro	TCG Ser	CAC His	CAT His	GCG Ala	GCG Ala	CAG Gln	TTC Phe	CCC Pro	AAC Asn	CAC His	902
TCA Ser	TTC Phe	AAG Lys	CAT His	GAG Glu	GAT Asp	CCC Pro	ATG Met	GGC Gly	CAG Gln	CAG Gln	GGC Gly	TCG Ser	941
CTG Leu	GGT Gly	GAG Glu	CAG Gln	CAG Gln	TAC Tyr	TCG Ser	GTG Val	CCG Pro	CCC Pro	CCG Pro	GTC Val	TAT Tyr	980
GGC Gly	TGC Cys	CAC His	ACC Thr	CCC Pro	ACC Thr	GAC Asp	AGC Ser	TGC Cys	ACC Thr	GGC Gly	AGC Ser	CAG Gln	1019
GCT Ala	TTG Leu	CTG Leu	CTG Leu	AGG Arg	ACG Thr	CCC Pro	TAC Tyr	AGC Ser	AGT Ser	GAC Asp	AAT Asn	TTA Leu	1058
TAC Tyr	CAA Gln	ATG Met	ACA Thr	TCC Ser	CAG Gln	CTT Leu	GAA Glu	TGC Cys	ATG Met	ACC Thr	TGG Trp	AAT Asn	1097
CAG Gln	ATG Met	AAC Asn	TTA Leu	GGA Gly	GCC Ala	ACC Thr	TTA Leu	AAG Lys	GGA Gly	CAC His	AGC Ser	ACA Thr	1136
GGG Gly	TAC Tyr	GAG Glu	AGC Ser	GAT Asp	AAC Asn	CAC His	ACA Thr	ACG Thr	CCC Pro	ATC Ile	CTC Leu	TGC Cys	1175
GGA Gly	GCC Ala	CAA Gln	TAC Tyr	AGA Arg	ATA Ile	CAC His	ACG Thr	CAC His	GGT Gly	GTC Val	TTC Phe	AGA Arg	1214

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GGC Gly	ATT Ile 280	CAG Gln	GAT Asp	GTG Val	CGA Arg	CGT Arg 285	GTG Val	CCT Pro	GGA Gly	GTA Val	GCC Ala 290	CCG Pro	1253
ACT Thr	CTT Leu	GTA Val	CGG Arg 295	TCG Ser	GCA Ala	TCT Ser	GAG Glu	ACC Thr 300	AGT Ser	GAG Glu	AAA Lys	CGC Arg	1292
CCC Pro 305	TTC Phe	ATG Met	TGT Cys	GCT Ala	TAC Tyr 310	CCA Pro	GGC Gly	TGC Cys	AAT Asn	AAG Lys 315	AGA Arg	TAT Tyr	1331
TTT Phe	AAG Lys	CTG Leu 320	TCC Ser	CAC His	TTA Leu	CAG Gln	ATG Met 325	CAC His	AGC Ser	AGG Arg	AAG Lys	CAC His 330	1370
ACT Thr	GGT Gly	GAG Glu	AAA Lys 335	CCA Pro	TAC Tyr	CAG Gln	TGT Cys	GAC Asp	TTC Phe 340	AAG Lys	GAC Asp	TGT Cys	1409
GAA Glu	CGA Arg 345	AGG Arg	TTT Phe	TCT Ser	CGT Arg	TCA Ser 350	GAC Asp	CAG Gln	CTC Leu	AAA Lys 355	AGA Arg	CAC His	1448
CAA Gln	AGG Arg	AGA Arg	CAT His 360	ACA Thr	GGT Gly	GTG Val	AAA Lys 365	CCA Pro	TTC Phe	CAG Gln	TGT Cys	AAA Lys	1487
ACT Thr 370	TGT Cys	CAG Gln	CGA Arg	AAG Lys	TTC Phe 375	TCC Ser	CGG Arg	TCC Ser	GAC Asp	CAC His 380	CTG Leu	AAG Lys	1526
ACC Thr	CAC His 385	ACC Thr	AGG Arg	ACT Thr	CAT His	ACA Thr	GGT Gly 390	GAA Glu	AAG Lys	CCC Pro	TTC Phe	AGC Ser 395	1565
TGT Cys	CGG Arg	TGG Trp	CCA Pro	AGT Ser 400	TGT Cys	CAG Gln	AAA Lys	AAG Lys	TTT Phe 405	GCC Ala	CGG Arg	TCA Ser	1604
GAT Asp	GAA Glu 410	TTA Leu	GTC Val	CGC Arg	CAT His	CAC His 415	AAC Asn	ATG Met	CAT His	CAG Gln	AGA Arg 420	AAC Asn	1643
ATG Met	ACC Thr	AAA Lys	CTC Leu 425	CAG Gln	CTG Leu	GCG Ala	CTT Leu	TGAGGGGTCT	CCC				1680

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 429 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala
 1             5             10
Val Pro Ser Leu Gly Gly Gly Gly Cys Ala Leu Pro Val
15             20             25
Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe Ala Pro
30             35             40
Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro
45             50             55
Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro His Ser
60             65             70
Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His
75             80
Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly
85             90             95
Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
100            105            110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg
115            120            125
Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser
130            135            140
Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe
145            150
Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His His Ala
155            160            165
Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro Met
170            175            180
Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro
185            190            195

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Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr
 200 205 210
 Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
 215 220
 Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp
 225 230 235
 Asn Gln Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr
 240 245 250
 Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly
 255 260 265
 Ala Gln Tyr Arg Ile His Thr His Gly Val Phe Arg Gly Ile
 270 275 280
 Gln Asp Val Arg Arg Val Pro Gly Val Ala Pro Thr Leu Val
 285 290
 Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg Pro Phe Met Cys
 295 300 305
 Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys Leu Ser His
 310 315 320
 Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro Tyr
 325 330 335
 Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Ser Arg Ser
 340 345 350
 Asp Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys
 355 360
 Pro Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser
 365 370 375
 Asp His Leu Lys Thr His Thr Arg Thr His Thr Gly Glu Lys
 380 385 390
 Pro Phe Ser Cys Arg Trp Pro Ser Cys Gln Lys Lys Phe Ala
 395 400 405
 Arg Ser Asp Glu Leu Val Arg His His Asn Met His Gln Arg
 410 415 420
 Asn Met Thr Lys Leu Gln Leu Ala Leu
 425

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATGAGAGGA TCGCATCACC ATCACCATCA CTC

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATGGAGTGA TGGTGATGGT GATGCGATCC TCT

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Val	Ala	Ala	Gly	Ser	Ser	Ser	Ser	Val	Lys	Trp	Thr	Glu	Gly
1				5					10				

Gln	Ser	Asn
15		

WHAT IS CLAIMED IS:

1. A hybridoma cell line which produces a monoclonal antibody specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4.
2. The hybridoma cell line according to claim 1, wherein said cell line is ATCC No. 11598.
3. The hybridoma cell line according to claim 1, wherein said cell line is ATCC No. 11599.
4. The hybridoma cell line according to claim 1, wherein said cell line is ATCC No. 11560.
5. A monoclonal antibody directed against an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4, said antibody capable of specifically binding to Wilms' tumor protein.
6. The antibody according to claim 5, wherein said antibody is H2.
7. The antibody according to claim 5, wherein said antibody is H7.
8. The antibody according to claim 5, wherein said antibody is HC17.
9. A polypeptide derived from a monoclonal antibody directed against an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4, said polypeptide selected from the group consisting of
 - (a) heavy chain variable region polypeptides of said monoclonal antibody;

- (b) light chain variable region polypeptides of said monoclonal antibody;
- (c) a Fab fragment of said antibody;
- (d) a F(ab)₂ fragment of said antibody; and
- (e) an Fv fragment of said antibody.

10. A method for diagnosing a disease condition characterized by WT1 expression comprising the steps of:

- a) providing a biological sample from a patient having the clinical symptoms associated with mesothelioma;
- b) contacting said sample with a monoclonal antibody or functional fragment thereof specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4; and
- c) detecting the presence of binding of said monoclonal antibody or fragment to said biological sample, wherein the presence of such binding indicates the presence of said disease condition.

11. The method according to claim 10, wherein said antibody is selected from the group consisting of H2, H7, HC17, and a cocktail thereof.

12. The method according to claim 10, wherein said biological sample is selected from the group consisting of whole blood, serum, plasma, synovial fluid, and tissue and said disease condition is selected from the group consisting of mesothelioma, prostate cancer, ovarian cancer, and leukemia.

13. A method of monitoring therapy in leukemia patients comprising the steps of:

- a) providing a biological sample from a patient treated for leukemia;
- b) contacting said sample with a monoclonal antibody or functional fragment thereof specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4; and
- c) detecting the presence of binding of said monoclonal antibody or fragment thereof to said biological sample, wherein the presence of such binding indicates the presence of a active leukemia cells.

14. The method according to claim 13, wherein said antibody is selected from the group consisting of H2, H7, HC17, and a cocktail thereof.

15. The method according to claim 13, wherein said biological sample is selected from the group consisting of whole blood, plasma, serum, urine and bone marrow.

16. The use of a monoclonal antibody raised against an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4 in detecting a disease characterized by the expression of the Wilms' tumor antigen.

17. A kit for diagnosing a disease characterized by the expression of the Wilms' tumor antigen comprising a monoclonal antibody raised against an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4 and means for signal generation.

18. The kit according to claim 17 wherein said monoclonal antibody is selected from the group consisting of H2, H7 and HC17.

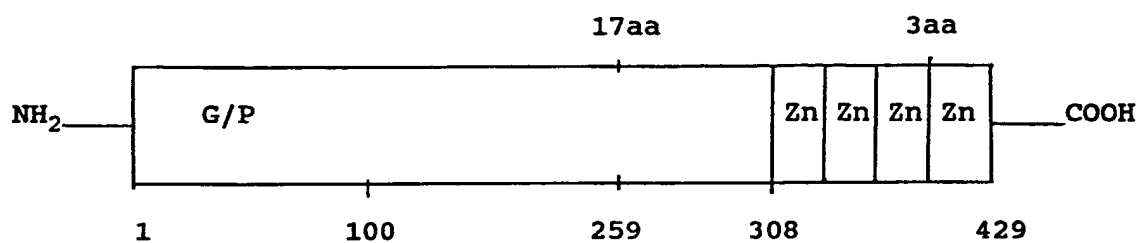
19. An antibody construct comprising at least one complementarity determining region from a monoclonal antibody specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4, said construct selected from the group consisting of a humanized antibody, a chimeric antibody, and a bi-specific antibody.

20. The antibody according to claim 19 wherein said monoclonal antibody is selected from the group consisting of H2, H7 and HC17.

21. A method for producing an antibody construct comprising employing at least one complementarity determining region or heavy chain variable region from a monoclonal antibody specific for an epitope located in Wilms' tumor protein antigen amino acids 1-181 SEQ ID NO: 4.

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FIGURE 1



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FIGURE 2A

ATG	AGA	GGA	TCG	CAT	CAC	CAT	CAC	CAT	CAC	TCC	ATG	GGT	TCC	42
Met	Arg	Gly	Ser	His	His	His	His	His	His	Ser	Met	Gly	Ser	
1				5					10					
GAC	GTT	CGT	GAC	CTG	AAC	GCA	CTG	CTG	CCG	GCA	GTT	CCG	TCC	84
Asp	Val	Arg	Asp	Leu	Asn	Ala	Leu	Leu	Pro	Ala	Val	Pro	Ser	
15					20					25				
CTG	GGT	GGT	GGT	GGT	GGT	TGC	GCA	CTG	CCG	GTT	AGC	GGT	GCA	126
Leu	Gly	Gly	Gly	Gly	Gly	Cys	Ala	Leu	Pro	Val	Ser	Gly	Ala	
	30					35					40			
GCA	CAG	TGG	GCT	CCG	GTT	CTG	GAC	TTC	GCA	CCG	CCG	GGT	GCA	168
Ala	Gln	Trp	Ala	Pro	Val	Leu	Asp	Phe	Ala	Pro	Pro	Gly	Ala	
		45					50					55		
TCC	GCA	TAC	GGT	TCC	CTG	GGT	GGT	CCG	GCA	CCG	CCG	CCG	GCA	210
Ser	Ala	Tyr	Gly	Ser	Leu	Gly	Gly	Pro	Ala	Pro	Pro	Pro	Ala	
			60					65					70	
CCG	CCG	CCG	CCG	CCG	CCG	CCG	CCG	CCG	CAC	TCC	TTC	ATC	AAA	252
Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	His	Ser	Phe	Ile	Lys	
				75					80					
CAG	GAA	CCG	AGC	TGG	GGT	GGT	GCA	GAA	CCG	CAC	GAA	GAA	CAG	294
Gln	Glu	Pro	Ser	Trp	Gly	Gly	Ala	Glu	Pro	His	Glu	Glu	Gln	
85					90					95				
TGC	CTG	AGC	GCA	TTC	ACC	GTT	CAC	TTC	TCC	GGC	CAG	TTC	ACT	336
Cys	Leu	Ser	Ala	Phe	Thr	Val	His	Phe	Ser	Gly	Gln	Phe	Thr	
	100					105					110			
GGC	ACA	GCC	GGA	GCC	TGT	CGC	TAC	GGG	CCC	TTC	GGT	CCT	CCT	378
Gly	Thr	Ala	Gly	Ala	Cys	Arg	Tyr	Gly	Pro	Phe	Gly	Pro	Pro	
		115					120					125		
CCG	CCC	AGC	CAG	GCG	TCA	TCC	GGC	CAG	GCC	AGG	ATG	TTT	CCT	420
Pro	Pro	Ser	Gln	Ala	Ser	Ser	Gly	Gln	Ala	Arg	Met	Phe	Pro	
			130					135					140	
AAC	GCG	CCC	TAC	CTG	CCC	AGC	TGC	CTC	GAG	AGC	CAG	CCC	GCT	462
Asn	Ala	Pro	Tyr	Leu	Pro	Ser	Cys	Leu	Glu	Ser	Gln	Pro	Ala	
				145					150					
ATT	CGC	AAT	CAG	GGT	TAC	AGC	ACG	GTC	ACC	TTC	GAC	GGG	ACG	504
Ile	Arg	Asn	Gln	Gly	Tyr	Ser	Thr	Val	Thr	Phe	Asp	Gly	Thr	
155					160					165				

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FIGURE 2B

CCC	AGC	TAC	GGT	CAC	ACG	CCC	TCG	CAC	CAT	GCG	GCG	CAG	TTC	546
Pro	Ser	Tyr	Gly	His	Thr	Pro	Ser	His	His	Ala	Ala	Gln	Phe	
	170					175					180			
CCC	AAC	CAC	TCA	TTC	AAG	CAT	GAG	GAT	CCG	GCT	GCT	AAC	AAA	588
Pro	Asn	His	Ser	Phe	Lys	His	Glu	Asp	Pro	Ala	Ala	Asn	Lys	
	185						190					195		
GCC	CGA	AAG	GAA	GCT	GAG	TTG	GCT	GCT	GCC	ACC	GCT	GAG	CAA	630
Ala	Arg	Lys	Glu	Ala	Glu	Leu	Ala	Ala	Ala	Thr	Ala	Glu	Gln	
			200				205						210	
TAA														633

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FIGURE 3A

GTTCAAGGCA GCGCCACAC CCGGGGGCTC TGC GCAACCC GACCGCCTGT	50
CCGCTCCCCC ACTTCCCGCC CTCCCTCCCA CCTACTCATT CACCCACCCA	100
CCCACCCAGA GCCGGGACGG CAGCCCAGGC GCCCGGGCCC CGCCGTCTCC	150
TCGCCGCGAT CCTGGACTTC CTCTTGCTGC AGGACCCGGC TTCCACGTGT	200
GTCCCGGAGC CGGCGTCTCA GCACACGCTC CGCTCCGGGC CTGGGTGCCT	250
ACAGCAGCCA GAGCAGCAGG GAGTCCGGGA CCCGGGCGGC ATCTGGGCCA	300
AGTTAGGCGC CGCCGAGGCC AGCGCTGAAC GTCTCCAGGG CCGGAGGAGC	350
CGCGGGGCGT CCGGGTCTGA GCCTCAGCAA ATG GGC TCC GAC GTG	395
Met Gly Ser Asp Val	
1 5	
CGG GAC CTG AAC GCG CTG CTG CCC GCC GTC CCC TCC CTG GGT	437
Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly	
10 15	
GGC GGC GGC GGC TGT GCC CTG CCT GTG AGC GGC GCG GCG CAG	479
Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln	
20 25 30	
TGG GCG CCG GTG CTG GAC TTT GCG CCC CCG GGC GCT TCG GCT	521
Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala	
35 40 45	
TAC GGG TCG TTG GGC GGC CCC GCG CCG CCA CCG GCT CCG CCG	563
Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro	
50 55 60	
CCA CCC CCG CCG CCG CCG CCT CAC TCC TTC ATC AAA CAG GAG	605
Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu	
65 70 75	
CCG AGC TGG GGC GGC GCG GAG CCG CAC GAG GAG CAG TGC CTG	647
Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln Cys Leu	
80 85	
AGC GCC TTC ACT GTC CAC TTT TCC GGC CAG TTC ACT GGC ACA	689
Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr	
90 95 100	
GCC GGA GCC TGT CGC TAC GGG CCC TTC GGT CCT CCT CCG CCC	731
Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro	
105 110 115	

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FIGURE 3B

AGC	CAG	GCG	TCA	TCC	GGC	CAG	GCC	AGG	ATG	TTT	CCT	AAC	GCG	773
Ser	Gln	Ala	Ser	Ser	Gly	Gln	Ala	Arg	Met	Phe	Pro	Asn	Ala	
		120					125					130		
CCC	TAC	CTG	CCC	AGC	TGC	CTC	GAG	AGC	CAG	CCC	GCT	ATT	CGC	815
Pro	Tyr	Leu	Pro	Ser	Cys	Leu	Glu	Ser	Gln	Pro	Ala	Ile	Arg	
			135					140					145	
AAT	CAG	GGT	TAC	AGC	ACG	GTC	ACC	TTC	GAC	GGG	ACG	CCC	AGC	857
Asn	Gln	Gly	Tyr	Ser	Thr	Val	Thr	Phe	Asp	Gly	Thr	Pro	Ser	
				150					155					
TAC	GGT	CAC	ACG	CCC	TCG	CAC	CAT	GCG	GCG	CAG	TTC	CCC	AAC	899
Tyr	Gly	His	Thr	Pro	Ser	His	His	Ala	Ala	Gln	Phe	Pro	Asn	
160					165					170				
CAC	TCA	TTC	AAG	CAT	GAG	GAT	CCC	ATG	GGC	CAG	CAG	GGC	TCG	941
His	Ser	Phe	Lys	His	Glu	Asp	Pro	Met	Gly	Gln	Gln	Gly	Ser	
	175					180					185			
CTG	GGT	GAG	CAG	CAG	TAC	TCG	GTG	CCG	CCC	CCG	GTC	TAT	GGC	983
Leu	Gly	Glu	Gln	Gln	Tyr	Ser	Val	Pro	Pro	Pro	Val	Tyr	Gly	
		190					195					200		
TGC	CAC	ACC	CCC	ACC	GAC	AGC	TGC	ACC	GGC	AGC	CAG	GCT	TTG	1025
Cys	His	Thr	Pro	Thr	Asp	Ser	Cys	Thr	Gly	Ser	Gln	Ala	Leu	
			205					210					215	
CTG	CTG	AGG	ACG	CCC	TAC	AGC	AGT	GAC	AAT	TTA	TAC	CAA	ATG	1067
Leu	Leu	Arg	Thr	Pro	Tyr	Ser	Ser	Asp	Asn	Leu	Tyr	Gln	Met	
				220					225					
ACA	TCC	CAG	CTT	GAA	TGC	ATG	ACC	TGG	AAT	CAG	ATG	AAC	TTA	1109
Thr	Ser	Gln	Leu	Glu	Cys	Met	Thr	Trp	Asn	Gln	Met	Asn	Leu	
230					235					240				
GGA	GCC	ACC	TTA	AAG	GGA	CAC	AGC	ACA	GGG	TAC	GAG	AGC	GAT	1151
Gly	Ala	Thr	Leu	Lys	Gly	His	Ser	Thr	Gly	Tyr	Glu	Ser	Asp	
	245					250					255			
AAC	CAC	ACA	ACG	CCC	ATC	CTC	TGC	GGA	GCC	CAA	TAC	AGA	ATA	1193
Asn	His	Thr	Thr	Pro	Ile	Leu	Cys	Gly	Ala	Gln	Tyr	Arg	Ile	
		260				265						270		
CAC	ACG	CAC	GGT	GTC	TTC	AGA	GGC	ATT	CAG	GAT	GTG	CGA	CGT	1235
His	Thr	His	Gly	Val	Phe	Arg	Gly	Ile	Gln	Asp	Val	Arg	Arg	
			275				280					285		
GTG	CCT	GGA	GTA	GCC	CCG	ACT	CTT	GTA	CGG	TCG	GCA	TCT	GAG	1277
Val	Pro	Gly	Val	Ala	Pro	Thr	Leu	Val	Arg	Ser	Ala	Ser	Glu	
				290				295						

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FIGURE 3C

ACC	AGT	GAG	AAA	CGC	CCC	TTC	ATG	TGT	GCT	TAC	CCA	GGC	TGC	1319
Thr	Ser	Glu	Lys	Arg	Pro	Phe	Met	Cys	Ala	Tyr	Pro	Gly	Cys	
300					305					310				
AAT	AAG	AGA	TAT	TTT	AAG	CTG	TCC	CAC	TTA	CAG	ATG	CAC	AGC	1361
Asn	Lys	Arg	Tyr	Phe	Lys	Leu	Ser	His	Leu	Gln	Met	His	Ser	
315					320					325				
AGG	AAG	CAC	ACT	GGT	GAG	AAA	CCA	TAC	CAG	TGT	GAC	TTC	AAG	1403
Arg	Lys	His	Thr	Gly	Glu	Lys	Pro	Tyr	Gln	Cys	Asp	Phe	Lys	
	330					335						340		
GAC	TGT	GAA	CGA	AGG	TTT	TCT	CGT	TCA	GAC	CAG	CTC	AAA	AGA	1445
Asp	Cys	Glu	Arg	Arg	Phe	Ser	Arg	Ser	Asp	Gln	Leu	Lys	Arg	
		345					350						355	
CAC	CAA	AGG	AGA	CAT	ACA	GGT	GTG	AAA	CCA	TTC	CAG	TGT	AAA	1487
His	Gln	Arg	Arg	His	Thr	Gly	Val	Lys	Pro	Phe	Gln	Cys	Lys	
				360				365						
ACT	TGT	CAG	CGA	AAG	TTC	TCC	CGG	TCC	GAC	CAC	CTG	AAG	ACC	1529
Thr	Cys	Gln	Arg	Lys	Phe	Ser	Arg	Ser	Asp	His	Leu	Lys	Thr	
370				375						380				
CAC	ACC	AGG	ACT	CAT	ACA	GGT	GAA	AAG	CCC	TTC	AGC	TGT	CGG	1571
His	Thr	Arg	Thr	His	Thr	Gly	Glu	Lys	Pro	Phe	Ser	Cys	Arg	
	385					390					395			
TGG	CCA	AGT	TGT	CAG	AAA	AAG	TTT	GCC	CGG	TCA	GAT	GAA	TTA	1613
Trp	Pro	Ser	Cys	Gln	Lys	Lys	Phe	Ala	Arg	Ser	Asp	Glu	Leu	
	400					405						410		
GTC	CGC	CAT	CAC	AAC	ATG	CAT	CAG	AGA	AAC	ATG	ACC	AAA	CTC	1655
Val	Arg	His	His	Asn	Met	His	Gln	Arg	Asn	Met	Thr	Lys	Leu	
		415					420					425		
CAG	CTG	GCG	CTT	TGAGGGGTCT	CCC									1680
Gln	Leu	Ala	Leu											

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>3</u> , line <u>32-33</u> of the description ¹	
A. IDENTIFICATION OF DEPOSIT ² Further deposits are identified on an additional sheet <input checked="" type="checkbox"/> ³	
Name of depositary institution ⁴ American Type Culture Collection	
Address of depositary institution (including postal code and country) ⁴ 12301 Parklawn Drive Rockville, Maryland 20852 USA	
Date of deposit ⁴ 31 March 1994	Accession Number ⁴ HB 11598
B. ADDITIONAL INDICATIONS ¹ (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ² (If the indications are not for all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS ² (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later ² (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
 (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau ¹⁰	
was (Authorized Officer)	

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 3, line 34-35 of the description ***A. IDENTIFICATION OF DEPOSIT ***Further deposits are identified on an additional sheet ☒ *

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit *

31 March 1994

Accession Number *

HB 11599

B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *** (If the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is *

was

(Authorized Officer)

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 4, line 1-2 of the description ***A. IDENTIFICATION OF DEPOSIT ***Further deposits are identified on an additional sheet ☒ *

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit *

31 March 1994

Accession Number *

HB 11600

B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *** (If the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau *

was

(Authorized Officer)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05523

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/12, 15/02; C12P 21/08; G01N 33/574; C07K 16/18, 16/30

US CL : 435/240.27, 172.2, 70.21, 7.23; 530/387.9, 387.3, 388.8, 388.85

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.27, 172.2, 70.21, 7.23; 530/387.9, 387.3, 388.8, 388.85

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: CA, BIOSIS, MEDLINE, EMBASE, CANCERLIT, BIOTECHDS, GENESEQ, SWISS-PROT, PIR, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ONCOGENE, VOLUME 6, NUMBER 12, ISSUED 12 DECEMBER 1991, MORRIS ET AL, "CHARACTERIZATION OF THE ZINC FINGER PROTEIN ENCODED BY THE WT1 WILMS' TUMOR LOCUS", PAGES 2339-2348, SEE ENTIRE DOCUMENT.	1-21
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCE, VOLUME 90, ISSUED JUNE 1993, MAHESWARAN ET AL, "PHYSICAL AND FUNCTIONAL INTERACTION BETWEEN WT1 AND p53 PROTEINS", PAGES 5100-5104, SEE ENTIRE DOCUMENT.	1-21
Y	WO 91/07509 (CALL ET AL) 30 MAY 1991, SEE ENTIRE DOCUMENT.	1-21

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

17 JULY 1995

Date of mailing of the international search report

03 AUG 1995

Name and mailing address of the ISA/US
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SUSAN A. LORING

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05523

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLINICAL CHEMISTRY, VOLUME 27, NUMBER 11, ISSUED 1981, SEVIER ET AL, "MONOCLONAL ANTIBODIES IN CLINICAL IMMUNOLOGY", PAGES 1797-1806, SEE ENTIRE DOCUMENT.	1-21
Y	NATURE, VOLUME 351, ISSUED 06 JUNE 1991, CO ET AL, "HUMANIZED ANTIBODIES FOR THERAPY", PAGES 501-502, SEE ENTIRE DOCUMENT.	19-21
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCE, VOLUME 81, ISSUED NOVEMBER 1984, MORRISON ET AL, "CHIMERIC HUMAN ANTIBODY MOLECULES: MOUSE ANTIGEN-BINDING DOMAINS WITH HUMAN CONSTANT REGION DOMAINS", PAGES 6851-6855, SEE ENTIRE DOCUMENT.	19-21
Y	US,A,5,141,736 (IWASA ET AL) 25 AUGUST 1992, SEE ENTIRE DOCUMENT.	19-21